vol 71
JOURNAL OF VIROLOGY, Feb. 1997, p. 1196–1206
0022-538X/97/$04.00+0

Second-Site Proviral Enhancer Alterations in Lymphomas Induced by Enhancer Mutants of SL3-3 Murine Leukemia Virus: Negative Effect of Nuclear Factor 1 Binding Site

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Received 15 July 1996/Accepted 6 November 1996

SL3-3 is a highly T-lymphomagenic murine retrovirus. Previously, mutation of binding sites in the U3 repeat region for the AML1 transcription factor family (also known as core binding factor [CBF], polyomavirus enhancer binding protein 2 [PEBP2], and SL3-3 enhancer factor 1 [SEF1]) were found to strongly reduce the pathogenicity of SL3-3 (B. Hallberg, J. Schmidt, A. Luz, F. S. Pedersen, and T. Grundström, J. Virol. 65:4177–4181, 1991). We have now examined the few cases in which tumors developed harboring proviruses that besides the AML1 (core) site mutations carried second-site alterations in their U3 repeat structures. In three distinct cases we observed the same type of alteration which involved deletions of regions known to contain binding sites for nuclear factor 1 (NF1) and the addition of extra enhancer repeat elements. In transient-expression experiments in T-lymphoid cells, these new U3 regions acted as stronger enhancers than the U3 regions of the original viruses. This suggests that the altered proviruses represent more-pathogenic variants selected for in the process of tumor formation. To analyze the proviral alterations, we generated a series of different enhancer-promoter reporter constructs. These constructs showed that the additional repeat elements are not critical for enhancer strength, whereas the NF1 sites down-regulate the level of transcription in T-lymphoid cells whether or not the AML1 (core) sites are functional. We therefore also tested SL3-3 viruses with mutated NF1 sites. These viruses have unimpaired pathogenic properties and thereby distinguish SL3-3 from Moloney murine leukemia virus.

Murine leukemia viruses (MLVs) are non-oncogene-encoding compact retroviruses that induce leukemias and hematopoietic tumors when injected into newborn mice (13, 18, 67). MLV-induced leukemogenesis is a multistep process thought to involve deregulation of the expression of cellular proto-oncogenes by insertional mutagenesis (67, 68). Numerous studies have shown that the retroviral enhancer in the U3 region is a major determinant of the latency and specificity of hematopoietic disease induction (8, 14, 22, 30, 33, 55, 59). A likely explanation for this is that a powerful enhancer in a given cell type, besides conferring a high replication rate, may allow the retrovirus to act as a strong insertional activator in that cell type (50, 53).

A conserved area in the U3 region of the MLVs, mostly found within direct tandem repeats (19), consists of densely packed binding sites for different transcription factors. These sequences constitute a transcriptional enhancer in which the overall composition of the interacting factors shapes the transcriptional profile of the virus. Thus small nucleotide variations in the individual binding sites in this area have been shown to confer variations in cell-specific expression and disease-inducing potential for several of the viruses (19, 22, 40, 52, 59). The existence of closely related MLV isolates with distinct enhancer-dependent behaviors has facilitated the determination of specific disease-inducing elements in the MLV genome. Moreover, PCR technology has now also made it possible to readily isolate whole sets of proviral structures from their hosts and study the dynamics of wild-type and mutated MLV genomes during disease development.

The SL3-3 MLV induces T-cell lymphomas with a mean latency of 2 to 6 months, depending on the mouse strain used (30, 31). SL3-3 was isolated from a T-lymphoma cell line of an AKR mouse and is believed to be derived from weakly pathogenic Akv, an endogenous MLV of the AKR strain (31, 51). Most of the enhancer function of SL3-3 is contained in the U3 region within a 72-bp direct repeat followed by a third repetition of 34 bp (21). Characterized binding sites for transcription factors in this repeat region include the helix-loop-helix proteins SEF2-1 (or E2-2) (12) and ALP1 (43, 44) the glucocorticoid receptor (GR) (9), nuclear factor 1 (NF1) (45), and the hematopoietic factors Ets1 (40), Myb (71), and AML1 (65, 66, 72). Of these factors, ALP1, GR, AML1, and c-Myb have been directly shown to activate the transcription of SL3-3 (9, 42, 71, 72).

The AML1-CBFβ transcription factor complex, also known as polyomavirus enhancer binding protein 2 (PEBP2) and core binding factor (CBF), is encoded by several recently cloned genes (5, 6, 32, 47, 69). As several names are currently in use for these factors, we shall refer to the transcription factor family here as the AML1 family of proteins for simplicity. AML1 is implicated through chromosomal rearrangements in several forms of acute myeloid leukemia in humans (34, 39, 46), and a murine AML1 gene is essential for normal hematopoietic development (48). AML1 binds to the enhancer core DNA sequence, found in many T-cell-specific genes and vi-

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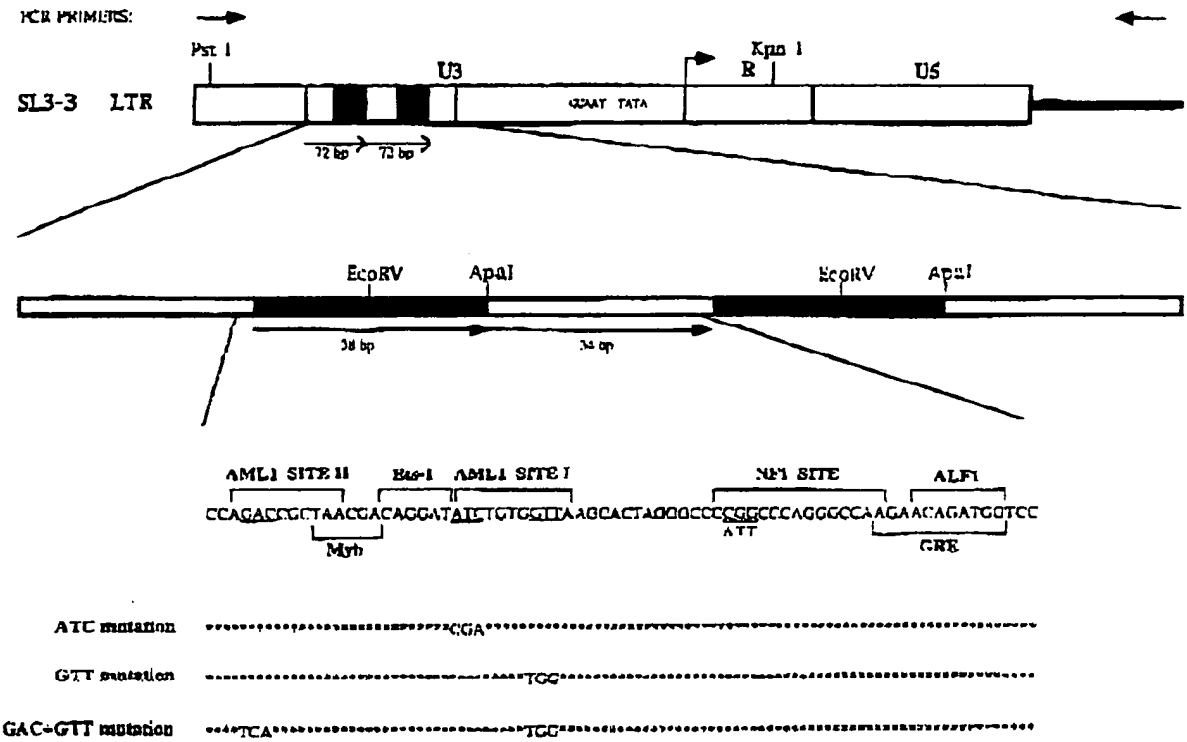


FIG. 1. Schematic representation of the structure of the SL3-3 LTR region with focus on the repeat structure found in the viral enhancer in the U3 region. The 34- and 38-bp repeat elements are represented by white and black boxes, respectively. This region contains two identical pairs of AML1 site I (core site) and AML1 site II (core site II) and three identical NF1 sites, besides known transcription factor binding sites for the factors Ets 1, Myb, ALF1, and GR (GRE) (see introduction for references). The positions of the different sets of mutations introduced into the AML1 sites in the study of Hallberg et al. (22) are shown in the lower part of the figure. The mutations were introduced into AML1 sites in both repeat elements. Also shown is the position of the CGG-to-ATT mutation used to render the NF1 site inactive. Restriction sites used for plasmid construction and the locations of the primers used for genomic PCR are indicated.

ruses, including all commonly known MLVs (19) and may act in synergy with members of the Myb and Ets transcription factor families (24, 62, 71). AML1 which we have previously referred to as SL3-3 enhancer factor 1 (SEF1) (65, 66) has two different binding sites which are both repeated once in the SL3-3 enhancer. AML1 activates the SL3-3 enhancer via these sites (72), and mutation of the AML1 binding sites has been found to reduce transient expression in T-lymphoid cells two- to fourfold (35, 65, 66), to strongly reduce the pathogenic potential (22, 40), and in the case of the related Moloney MLV, to alter the disease specificity and increase the latency period (59).

NF1 designates an ubiquitously expressed transcription factor family which is involved in the regulation of many cellular and viral genes (27). NF1 acts as a dimer and is encoded by four different genes in the mammalian genome which are all alternatively spliced, giving rise to a large set of NF1 complexes (4, 57). NF1 binding sites exist in one or two different forms in many but not all MLVs (19, 49). An NF1 binding site is repeated three times in the SL3-3 enhancer and has been shown by band-shift analysis to bind complexes very likely to be NF1 and to be important for transcription in several cell types (45). Mutation of the corresponding NF1 site in Moloney MLV was found to reduce transient expression in T-lymphoid cell lines (60) and substantially prolong the latency of disease induction (59).

We here report on alterations in the enhancer region of SL3-3 proviruses from murine tumors induced by SL3-3 carrying mutated core sites. These alterations do not involve the AML1 binding sites but the adjacent NF1 site. The altered U3 variants are stronger enhancers in T-lymphoid cells and may result from a selection for stronger and more-pathogenic enhancer structures compensating for the AML1 site mutations. Also, the NF1 sites act to stimulate transcription in fibroblasts, whereas they have a down-regulatory role on the SL3-3 reporter constructs in T-lymphoid cells. We also show that the NF1 sites are not critical for the pathogenicity of SL3-3.

MATERIALS AND METHODS

Cell culture. The murine T-lymphoid cell line L691 (15) was grown in RPMI 1640 medium containing Glutamax-1 (Gibco BRL, Life Technologies) and supplemented with 10% newborn calf serum and 100 U of penicillin per ml and 100 μg of streptomycin per ml. NIH 3T3 cells and the murine plasmacytoma B-cell line MPC11 (42) were grown in Dulbecco's modified Eagle medium containing Glutamax-1 (Gibco) and supplemented with 10% serum and antibiotics as described above.

Detection of sequences flanking the viral integration. Genomic sequences flanking the integrated SL3-3 proviral sequences in the tumor DNAs were PCR amplified as previously described by the method of Sørensen et al. (64).

Pathogenicity experiments. To generate SL3-3 viruses with mutated NF1 sites, the *Pst*I-*Kpn*I fragment of construct pESG130 (45) was inserted into a plasmid carrying the molecular clone of SL3-3 and infectious viruses produced by transfection of concatemerized *Pst*I-*Pst*I fragments into NIH 3T3 cells as previously described (22). Random bred NMRI strain mice which lack ecotropic endogenous proviruses (29) were used for pathogenicity assays. Tumor induction and classification of disease were done as previously described (22).

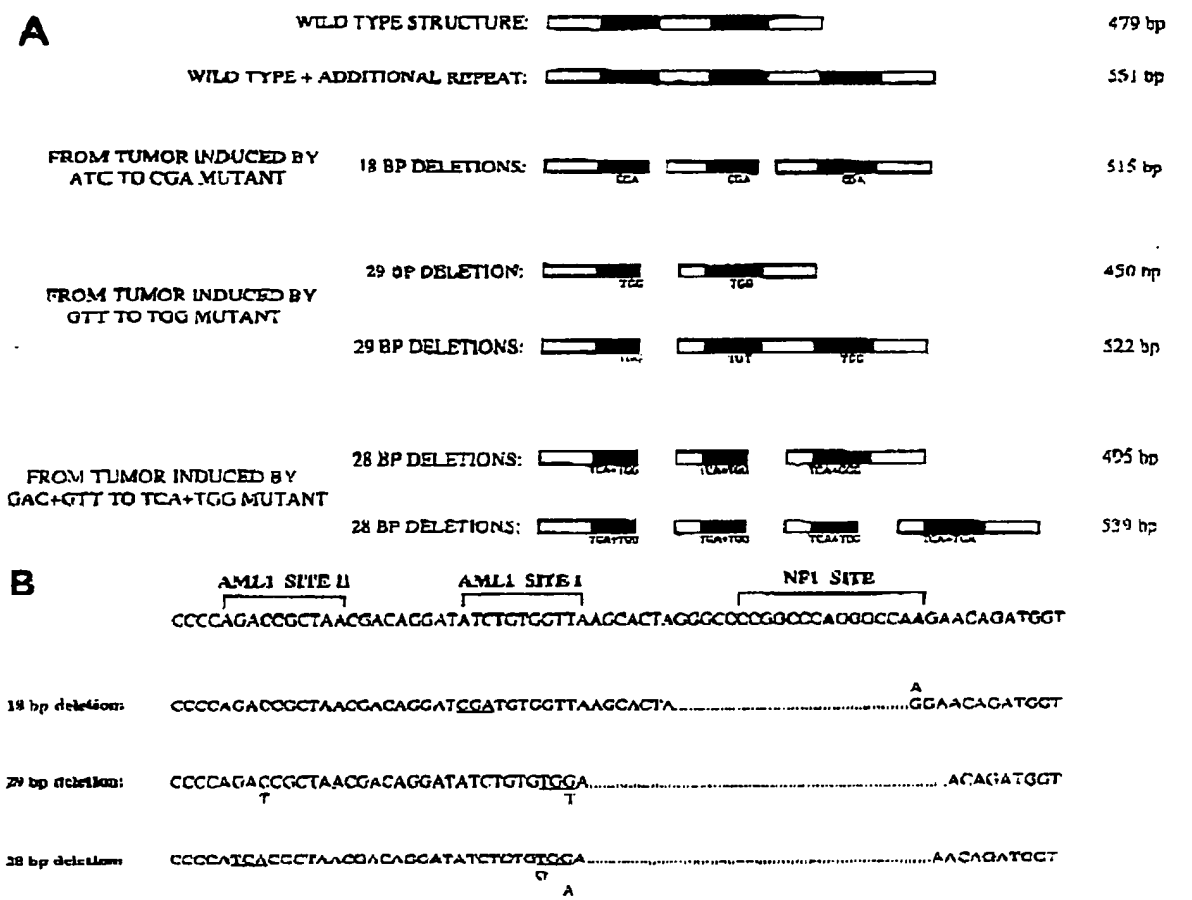


FIG. 2. Structures of the altered proviral enhancer regions found in the tumors of three different infected mice. The overall repeat structure (depicted schematically) (A) and the exact nucleotide sequences surrounding the deletions (B) are shown. In the tumor of a mouse infected with the ATC SL3-3 mutant, a structure with an 18-bp deletion and a concomitant base substitution was found. The GTT mutant gave rise to a tumor containing provirus with a 29-bp deletion in two versions, with or without an additional imperfect repeat element. The short version contained a base substitution in both AML1 sites II (indicated below the sequence). The longer version contained a base substitution in the central site of the three AML1 sites I. One mouse developed a tumor upon infection with the GAC-GTT mutant. Proviruses in this tumor contained a 28-bp deletion in versions with three or four repeat elements. Both had point mutations in the AML1 site I closest to the promoter. The short form contained a T-to-C base substitution, and the long form contained a G-to-A base substitution. The expected lengths of the *Pst*I-*Kpn*I fragments of the various types are indicated to the right.

PCR amplification of proviral DNA. DNA was prepared from frozen tumor material as previously described (22). PCR was performed by using a primer which recognizes the 5′ end of the U3 region, 5′-TTCATAAGGCTTAGCCAG CTAACTGCAG-3′, and a primer which recognizes a SL3-3-specific sequence outside the long terminal repeat (LTR) in the 5′ untranslated region, 5′-GAT GCCGGCACACACACACACACTCTCCC-3′ (see Fig. 1).

Plasmids. PCR-amplified U3 regions from the murine tumors were inserted into pL6CAT (36) from *Pst*I to *Kpn*I to generate constructs pSL3(TUMatcL)cat, pSL3(TUMgttL)cat, pSL3(TUMgttLL)cat, pSL3(TUMdmLL)cat, and pSL3(TUM dmXL)cat. Plasmids pSL3(gtt)cat, pSL3(atc)cat, and pSL3(dm)cat were made by inserting the *Pst*I-*Kpn*I fragments from the plasmid clones of the viruses carrying the ATC, GTT, and GAC-GTT mutations (22), respectively, into pL6CAT. Likewise, pSL3(3mNF1)cat was made by inserting the *Pst*I-*Kpn*I fragments from pESG130 (45) into pL6CAT. pSL3(1mNF1)cat was generated by insertion of the *Apa*I fragment from pSL3(3mNF1)cat into pSL3(WT)cat.

The remaining constructs were made by PCR mutagenesis (25). Construct pSL3(ΔBA), consisting of the SL3-3 LTR *Pst*I-*Kpn*I fragment containing an *Apa*I-*Apa*I 72-bp repeat element deletion inserted into pUC19 was used as template. PCR mutagenesis was performed with primer 5′-CGAGCTCGGTAC CCGGGCGACTCAGTCT-3′ combined with primer 5′-AACAGATGGTCCC CAGAAATAGCTAAAACAACAAC-3′ and with primer 5′-TTGCATGCCTG CAGTAACGCCATTTTGC-3′ combined with either primer 5′-TGTTTTAGC TATTTCTGGGGACCATCTGTTTAACCACAGATATCCTGTCG-3′ [pSL3 (Δ28)cat], primer 5′-TTAGCTATTTCTGGGGACCATCTGTTCCTTAGTGCTT AACCACAGATATCCT-3′ [pSL3(Δ18)cat], or primer 5′-TCTTTTAGCTATTTC TGGGGACCATCTGTTTCCACACAGATATCCTGTCG-3′ [pSL3(TUMdm-72)cat] in the latter case using the GAC-GTT mutant as the template instead of the wild type. In order to generate the two-repeat structure, constructs were cloned into pUC19 and *Ava*II fragments were ligated, cut with *Eco*RV, and ligated back into the *Eco*RV site in the parental construct. *Pst*I-*Kpn*I fragments were then inserted in pL6CAT, thereby generating pSL3(Δ18)cat and pSL3(Δ28)cat.

Plasmids pSL3(Δ18+72)cat and pSL3(Δ28+72)cat were made by cutting pSL3(Δ18)cat and pSL3(Δ28)cat, respectively, with *Pst*I and partially with *Eco*RV and inserting the gel-purified 186- and 196-bp fragments into pSL3 (ΔBA). The resulting plasmids were cut with *Kpn*I and partially with *Eco*RV to give fragments of 312 and 322 bp. The 186- and 196-bp and the 312- and 322-bp fragments were then concomitantly inserted into the *Pst*I-*Kpn*I sites of pL6CAT. pSL3(WT+72)cat was built the same way, based on the wild-type construct.

The nucleotide sequences of all constructs were verified by sequencing.

Sequence comparisons. Nucleotide sequences were compared with sequences in the GenBank (release 96.0 [August 1996]) and EMBL (release 48.0 [September 1996]) databases by using the Fasta program from the Wisconsin Package

EGCG [version 8.1.0 (a), May 1006 by Genetics Computer Group, Madison, Wis., and Peter Rice, The Sanger Centre, Cambridge, England]. Transcription factor sites were searched for with the FindPatterns program from the Wisconsin Package.

Southern blotting and hybridization. To detect the number of viral integrations of each tumor, Southern blotting was performed as previously described (63). Briefly, the genomic tumor DNA was cut with *HindIII*, which cuts once within the SL3-3 proviral genome, resolved on 0.75% agarose gel, and transferred to a nylon membrane. An ecotrope-specific probe consisting of 230 bp from the *env* region of the Akv retrovirus was ^{32}P labeled and used for hybridization (63). Clonal *c-myc* gene rearrangements in tumors were detected by a 635-bp probe of the *c-myc* promoter region (62).

For detection of the size of the variant U3 structures, genomic tumor DNA was digested with *PstI* and *KpnI*. Ten micrograms of DNA was resolved on a 1.5% agarose gel in 1× TBE (Tris-borate-EDTA) buffer and transferred onto a nylon membrane as described above. A probe specific for the SL3-3 repeat region was generated by primer extension of primer 5′-TTGAGACAGTTTCT GGGTCTCTTGA-3′ using ^{32}P-labeled dATPs and a purified *RsaI-KpnI* SL3-3 LTR fragment as the template. The probe did not cross-hybridize to sequences of the NMRI mouse genome.

Transfections and reporter assays. L691 cells were transfected by the DEAE-dextran method as previously described (50). Four micrograms of the various chloramphenicol acetyltransferase (CAT) constructs was used to transfect 5×10^6 cells at a density of 5×10^5 cells per ml. Most of the transfection series included 1 μg of pRSV-LUC (Promega) internal control plasmid to correct for variations in transfection efficiencies. The internal control plasmid did not influence the CAT values. NIH 3T3 and MPC11 cells were transfected by calcium phosphate-mediated precipitation (36) using 3 μg of the various CAT constructs and 0.75 μg of pCH110 (*lacZ* gene driven by the simian virus 40 early promoter) (36). CAT assays were performed by the method of Gorman (50), except that quantification of the radioactive spots was performed on the PhosphorImager. β-Galactosidase activity was measured by an *o*-nitrophenyl-β-D-galactopyranoside assay (36), and luciferase activity was measured with a luminometer (Berthold LB 9501) using the Luciferase Assay System from Promega (catalog no. E1501). All transfections were done in duplicate or triplicate and repeated between two and six times.

Nucleotide sequence accession numbers. The nucleotide sequences of the provirus-flanking sequences have been deposited in the EMBL data bank. The EMBL accession numbers are Y09129, Y09130, Y09131, Y09132, Y09133, Y09134, and Y09135.

RESULTS

Characterization of variable proviral U3 regions of mutant SL3-3-induced lymphomas. The tumor material we have investigated originates from an earlier study (22) in which several series of SL3-3 with mutated AML1 sites were injected into newborn NMRI mice (Fig. 1). All animals (49 mice) infected with wild-type SL3-3 developed lymphomas with a mean latency of 70 days. Mutating the AML1 site I (the core site, previously also termed SEF1 site I) resulted in lower incidences and longer latencies of disease. Thus, SL3-3 viruses carrying the ATC-to-CGA mutation or the GTT-to-TGG mutation induced lymphomas in 47% (9 of 19 mice) or 61% (28 of 46 mice) of the mice, respectively, within a 300-day observation period. SL3-3 mutated in both AML1 site I and AML1 site II (core site II, previously also termed SEF1 site II) was essentially nonpathogenic. Only 5% of mice infected with these viruses (1 of 19 mice) developed lymphomas within 300 days (22).

U3 regions of proviruses from tumors isolated from the mice were subsequently PCR amplified and sequenced. The introduced AML1 site mutations had not reverted (22). Some tumors contained proviruses with a few point mutations, but no coherent pattern could be recognized (2, 22). Several tumors contained proviruses that had lost one or gained one or two 72-bp direct repeats but otherwise were normal (see Discussion).

Apart from these changes, three tumors were found to contain altered proviral U3 regions. These tumors came from mice infected with SL3-3 of one of each of the ATC, GTT, and GAC-GTT mutation series. The tumors were all T-cell lymphomas, as judged by histological observations. The *PstI-KpnI* fragments (Fig. 1) of the 5′-LTR regions of these altered proviral structures were PCR cloned into the bacterial plasmid pL6CAT and sequenced. Cloning and sequencing of this region were done several times on the basis of independent PCRs to avoid PCR artifacts. The three altered U3 regions all have small deletions between AML1 site I and site II. The deletions comprise the NF1 binding site. Also, the U3 regions carry additional 72-bp direct repeats without deletions.

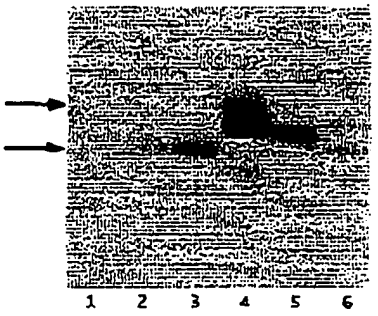


FIG. 3. Southern blot showing the lengths of the altered proviral structures in genomic DNAs from murine tumors. Genomic tumor DNA was cut with *PstI* and *KpnI*, resolved in a 1.5% agarose gel, transferred to a nylon membrane, and hybridized to a probe recognizing the SL3-3 enhancer repeat region. The lengths of the fragments correspond to the lengths expected from the PCR amplifications of the various proviral structures from the tumors. Lane 1 contains DNA from a tumor from an uninfected control mouse. Lanes 2 and 3 contain proviral DNAs from tumors induced by wild-type SL3-3 and included to indicate the fragment sizes. The first of these tumors contains an enhancer repeat triplicated provirus in addition to the wild-type length integration, both of which are indicated by arrows. Lanes 4, 5, and 6 are from the GTT, ATC, and GAC-GTT tumors respectively. The expected lengths of the fragments are indicated in Fig. 2.

The structures of the altered viral enhancers are shown in Fig. 2. The altered ATC mutant was found in the thymic tumor of a mouse deceased after 224 days. It contained an extra 72-bp repeat element and two identical 18-bp deletions combined with a base pair substitution in the two repeat elements distal to the promoter. The altered GTT mutants were found in a thymic tumor from a mouse at 272 days. This tumor contained four different viral structures. A provirus with a 29-bp deletion in the repeat region most distal to the promoter and two point mutations was found with and without an additional 72-bp repeat element. Also, an otherwise unaltered GTT mutant was found with or without the extra repeat (not shown in Fig. 2). The altered GAC-GTT mutant was found in a tumor in a lymph node of a mouse deceased at 190 days. It had an additional repeat element and a point mutation and carried two identical deletions of 28 bp in the two distal repeat elements. In this tumor we also found another altered U3 region which differed from the one mentioned above only in having a fourth repeat unit with the 28-bp deletion and a separate point mutation.

In order to eliminate the possibility that the proviral alterations arose as a result of the PCR, we performed Southern blotting on the genomic tumor DNA. We used the restriction enzymes *PstI* and *KpnI*, thereby cutting at both ends of the LTR region, and a probe specific for the SL3-3 repeat region (Fig. 3). The lengths of the various U3 regions seen on the Southern blot correspond to the lengths of the PCR products. The possibility also exists that we had PCR amplified endogenous sequences and not viral structures evolved from the exogenous mutant SL3-3 viruses. This, however, is highly unlikely since the altered structures all contain the originally introduced AML1 site mutations. Also, we have been unable

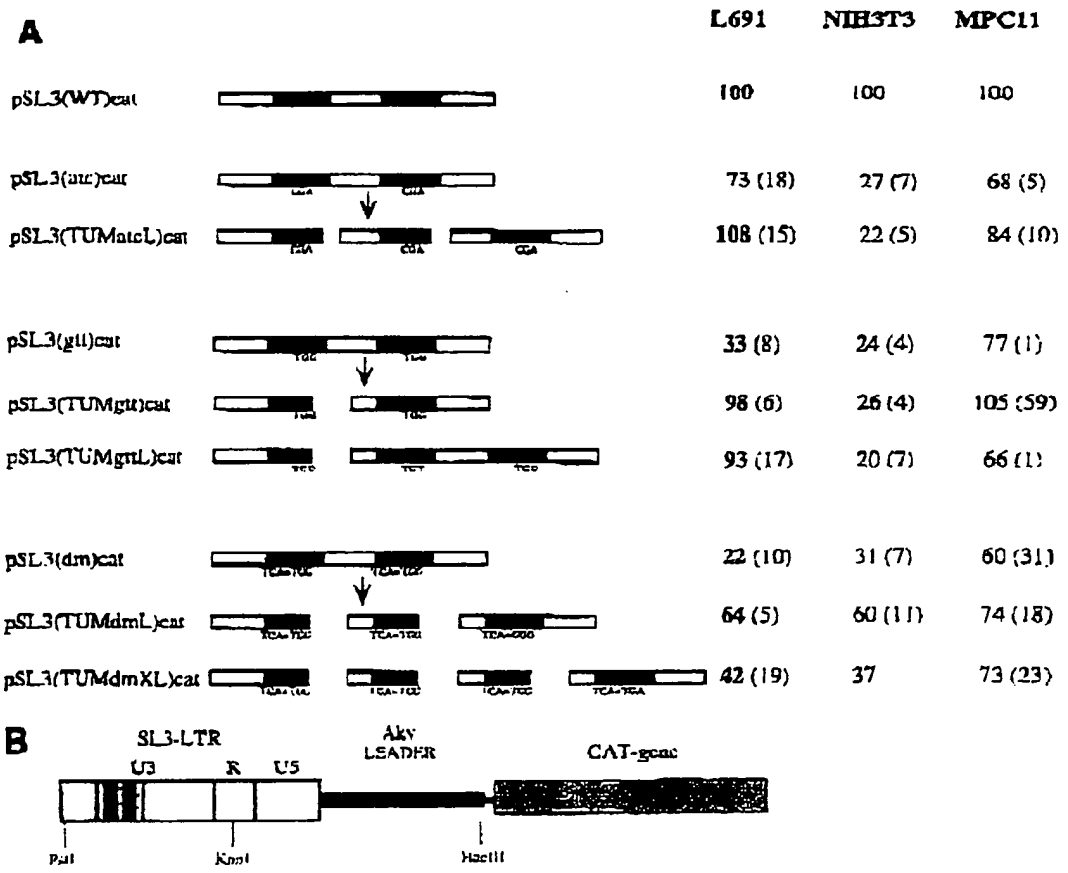


FIG. 4. (A) Transient-transfection assays with CAT reporter constructs. Transfections were done in series with each construct included two or three times. Within each series, the mean of the values for each construct was normalized to the wild-type level, which was arbitrarily set at 100. Standard deviations of two to six independent transfection series are given in parentheses. Arrows symbolize the evolution of the tumor-derived structures from their parent mutant viruses. pSL3(TUMdmXL)cat was tested in only one series in NIH 3T3 cells. (B) Schematic drawing of the reporter construct used for the CAT assays.

to amplify proviral structures from DNA preparations from uninfected mice (data not shown).

To further characterize the viral integrations, we performed Southern blotting of the tumor DNA using an ecotrope-specific SL3-3 probe. The results indicated that the DNAs contained different numbers of integrated proviruses as follows: ATC tumor, four; GTT tumor, five; and GAC-GTT tumor, two (data not shown). We then performed PCR amplification of the flanking regions of the integrated SL3-3 proviral sequences by the method of Sørensen et al. (64). Sequences of between 250 to 400 nucleotides of the genomic DNA flanking several of the proviral integration sites were obtained by this method. Database searches revealed that the altered ATC mutant was integrated in reverse orientation in the *c-myc* promoter region 698 nucleotides upstream of exon 1. Southern blotting with a *c-myc*-specific probe indicated that the tumor was clonal with respect to this integration (data not shown). None of the other detected proviral flanking regions matched sequences found in the GenEMBL database (the sequences were deposited in the EMBL databank).

The altered U3 regions are stronger enhancers in T-lymphoid cells. The altered U3 structures found (although different) all seemed to be altered according to the same theme, since they all had deletions of an area encompassing the NF1 site besides having variable numbers of repeat elements. This fact pointed to the notion that the altered viral enhancer structures could have played a role in the tumorigenic process.

To test if the alterations found in the SL3-3 enhancer region affected the enhancer strength, the *PstI-KpnI* LTR-fragments containing each of the altered U3 regions were cloned into the plasmid pL6CAT (36) such that each variant SL3-3 LTR region directs the expression of the CAT gene (Fig. 4B). Likewise, each of the parent AML1 site mutated U3 regions was inserted into pL6CAT.

The CAT plasmids were transfected into the murine T-lymphoid cell line L691. This cell line was chosen because measurements of transient-transcription levels in L691 cells have previously shown to correlate well with the actual pathogenicities of various SL3-3 AML1 site variants (22, 65). Measurements of transient CAT expression levels showed that the

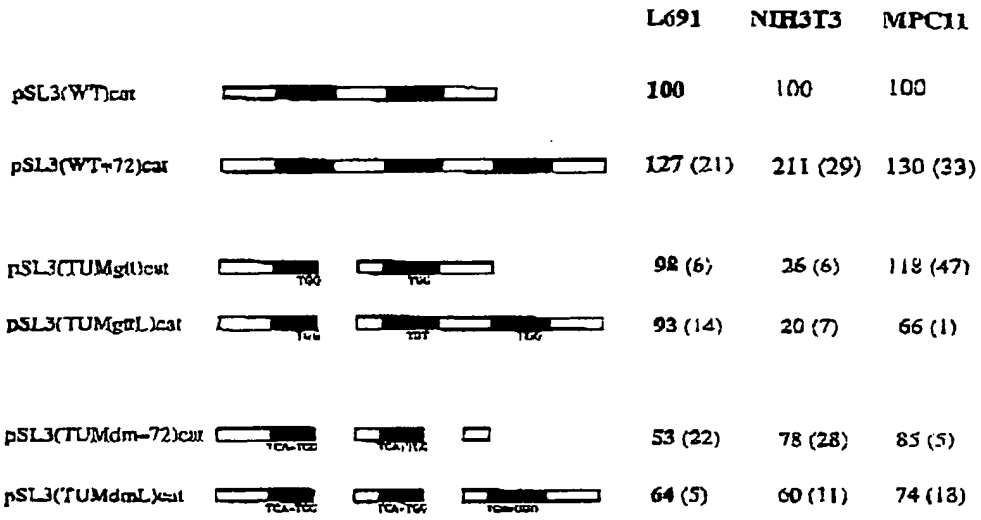


FIG. 5. Results of transient-transfection experiments designed to monitor the effect of the additional repeat elements. Values are as described in the legend to Fig. 4.

altered enhancer structures were reproducibly 1.5- to 3.5-fold more active than their parent structures (Fig. 4). These results support the hypothesis that among mutated viruses, more-tumorigenic virus variants are selected for in the process of tumor development.

T cells are the target cells for SL3-3, but the altered structures could also have an effect in other cell types. We therefore transfected the CAT constructs into two lymphoid cell lines and into fibroblasts. The murine plasmacytoma B-cell line MPC11 and NIH 3T3 cells did not show stronger enhancer activity of the altered proviral structures, indicating that these are particularly effective in T-lymphoid cells (Fig. 4).

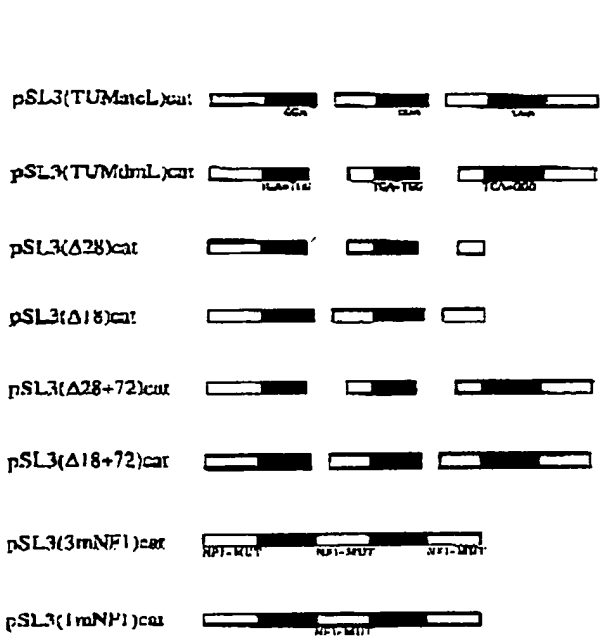
Limited effects of the additional repeat elements in target cells. In order to further investigate which parts of the altered proviral U3 regions contributed to making them stronger enhancers, we made a series of reporter constructs. These were designed to separate the effects of the different changes observed, i.e., the various numbers of 72-bp repeat units, the small and large deletions, the few point mutations, and the effect of abolishing the NF1 sites. All constructs were transfected into L691, NIH 3T3, and MPC11 cells, and transient CAT expression levels were measured.

All tumors contained viral U3 variants with an additional 72-bp repeat element. The increased enhancer effect could be a result of this, since increasing enhancer strength could simply be a matter of increasing the number of transcription factor binding sites. However, pSL3(TUMgttL)cat is not more active than pSL3(TUMgtt)cat (Fig. 5). Likewise, deleting the additional repeat element from the tumor-derived structure pSL3(TUMdmL)cat did not change the transcriptional level, as seen when comparing this construct with the construct pSL3(TUMdmL-72)cat. Indeed, the fourth repeat element on pSL3(TUMdmXL)cat actually reduces the enhancer strength (Fig. 4). Also, construct pSL3(WT+72)cat, which is similar to the wild-type construct except for the presence of an additional 72-bp repeat element, did not notably increase CAT activity over the wild-type level in L691 cells, although it was twice as active in NIH 3T3 cells. Therefore, the additional repeat elements seem to be of little importance for the increased enhancer strength of the altered viral structures.

The NF1 site has a down-regulatory role. We now asked if the function of the deletions was dependent on the AML1 site mutations. Since the AML1 sites are of major importance for the SL3-3 enhancer strength, a reversion of the AML1 site mutations in the altered structures should increase the transcriptional level if the function of the deletions did not depend on the presence of mutated AML1 sites. We chose to make constructs having wild-type AML1 sites and either the 18-bp deletion found in the altered ATC variant or the 28-bp deletion found in the altered GAC-GTT variant (Fig. 6). These deletions were made in each of the two 72-bp repeat units of the pSL3(WT)cat construct, thereby creating constructs pSL3(Δ28)cat and pSL3(Δ18)cat. In addition, we made constructs pSL3(Δ28+72)cat and pSL3(Δ18+72)cat, which besides two deletions carry a third undeleted repeat unit, thus being the equivalent forms of the tumor-derived structures pSL3(TUMatcL)cat and pSL3(TUMdmL)cat.

As seen when comparing pSL3(TUMatcL)cat with pSL3(Δ18+72)cat and pSL3(TUMdmL)cat with pSL3(Δ28+72)cat, the reconstitution of functional AML1 binding sites in the tumor-derived structures increases the enhancer strength. The slightly larger effect seen in the latter case probably reflects the larger effect obtained when reverting the more powerful GAC-GTT mutation compared to the ATC mutation. These constructs also support the conclusion that the extra repeat element does not play a significant role in increasing the enhancer strength.

It is possible that new transcription factor binding sites were created by the deletions. However, we have not been able to identify any known binding sites by database searches (see Materials and Methods). Also, the fact that the three observed deletions are not identical argues against this possibility. A more likely explanation therefore is that the deletions removed binding sites for interacting proteins. To investigate if the effect of the deletions was to abolish the NF1 binding site, we tested whether or not constructs without deletions but with



	L691	NIH3T3	MPC11
pSL3(TUMmcL)cat	108 (15)	22 (5)	84 (10)
pSL3(TUMdmL)cat	64 (5)	60 (11)	74 (18)
pSL3(Δ28)cat	120 (23)	41 (32)	134 (9)
pSL3(Δ18)cat	154 (30)	83 (33)	161 (18)
pSL3(Δ28+72)cat	127 (10)	189 (1)	170 (16)
pSL3(Δ18+72)cat	133 (10)	247 (11)	233 (11)
pSL3(3mNF1)cat	218 (13)	17 (7)	59 (3)
pSL3(1mNF1)cat	203 (10)	102 (23)	107 (1)

FIG. 6. Results of transient-transfection experiments designed to monitor the importance of the observed deletions and the effect of mutating the NF1 sites.

mutated NF1 sites also gave enhanced transcription levels. A mutation of three consecutive base pairs from GCC to ATT in the NF1 site has previously been found to abolish the binding of NF1 to this site in band-shift assays (45). Construct pSL3(3mNF1)cat which carries this mutation in all three NF1 sites gives a twofold increase in transient CAT levels over that of the wild-type construct in L691 cells (Fig. 6). Another construct, pSL3(1mNF1)cat, which carries the mutation only in the single central NF1 site, gives almost as large an increase. Thus, the NF1 site has a down-regulatory effect on the transcriptional level in the T-lymphoid cell line, and mutating just one of the three sites is sufficient to generate most of the observed effect. However, in NIH 3T3 cells, the transcriptional level of pSL3(3mNF1)cat is five times lower than the wild-type level, whereas the level of pSL3(1mNF1)cat is unaltered relative to the wild-type level. Thus, the NF1 binding site has two opposite functions in these two cell types: it is a positive regulator in fibroblasts and a negative regulator in T cells. Also, the fact that mutating only the single central site of the three NF1 sites gives an effect in T cells but is not sufficient for creating an effect in fibroblasts points to different usage of the sites in the two different cell types.

The NF1 sites do not contribute to the pathogenicity of SL3-3. The fact that the NF1 sites appeared to have a down-regulatory effect on the enhancer strength of SL3-3 in T cells was somewhat surprising, since mutation of the corresponding site in Moloney MLV has previously been found to reduce transient expression in T-lymphoid cells and to have a marked influence on the latency of disease induction, increasing it from 12 to 18 weeks on average (59). We therefore analyzed viruses carrying the CGG-to-ATT mutation in all three NF1 sites, i.e., having the same LTR regions as construct pSL3(3mNF1)cat. The viruses were generated by transfecting molecular clones of the viruses into NIH 3T3 cells. In line with the results from the CAT assays, the cells transfected with the NF1-mutated virus clones gave substantially lower titers than cells transfected with the wild-type clones. Similar observations have been made with NF1-mutated Moloney MLV (60). Equal amounts of SL3-3 with mutant or wild-type NF1 sites were injected into 24 and 49 newborn NMRI mice, respectively. All mice developed lymphomas within 180 days (Fig. 7). The SL3-3 viruses with NF1 mutations did not show altered disease-inducing abilities. Histological examination of the mice showed that the mutated viruses primarily induced thymic lymphomas, as did the wild-type viruses (22). The NF1-mutated SL3-3 did not exhibit prolonged latency or reduced incidence of disease induction compared to the wild-type virus. Sequencing of the NF1-mutated proviruses from the tumors did not show any unusual structures. These data clearly show that the NF1 binding sites are not important for the pathogenic potential of SL3-3, which means that SL3-3 and Moloney MLV behave differently with respect to the NF1 binding site.

DISCUSSION

MLV isolates may have quite distinct disease-inducing abilities, although they are similar in genomic organization. The oncogenic viruses are generally derived from tumors presumably induced by the viruses and are thus likely to be highly selected for disease induction. However, if the pathogenic determinants in the viral genome are weakened, the viruses would in all likelihood undergo selection for variants with reconstituted or otherwise different disease-inducing abilities. Mutations and rearrangements are known to happen at a high rate in the MLV genome (11). If only a few alterations are needed to produce highly pathogenic virus variants, these may occasionally evolve during the pathogenic process. The most potent of such variants would then be expected to be found in

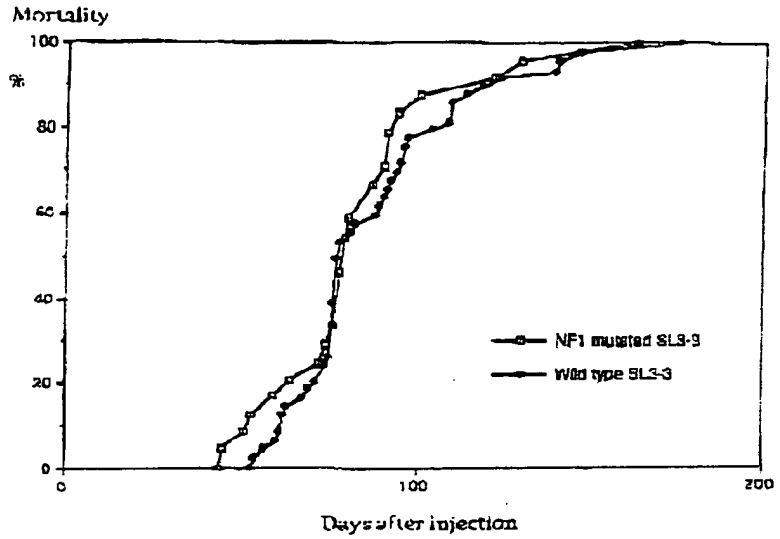


FIG. 7. Mortality curves of NMRI mice infected with either wild-type SL3-3 or SL3-3 with mutated NF1 sites. Twenty-four mice were infected with NF1 mutant virus, 49 mice were infected with wild-type SL3-3, and 17 mice were mock infected. The cumulative incidence of disease is shown against the number of days after injection and infection of the mice for a 200-day period. No control mice developed disease within this period.

the arising tumors. The study of the evolution of mutant viruses could provide insight in the mechanisms involved in induction of the various malignancies provoked by the MLVs as well as in the stepwise evolution of the new MLV variants.

In this study we have investigated lymphomas from mice infected with SL3-3 viruses whose pathogenic potential had been weakened by introduced mutations in the AML1 binding sites. We did not find cases in which functional AML1 sites had been recreated. However, in a related study in which only 1 bp in the SL3-3 AML1 site I was mutated, frequent reversions of the introduced mutation were found (40). This difference most likely reflects the lower probability of reconstituting 3-bp changes of a binding site, a point which may be of interest when designing mutations for future experiments.

In 3 of 84 tumors investigated, we found second-site deletions in the viral enhancers of proviruses from tumors induced by SL3-3 with mutated AML1 sites. Although we have no direct proof that these deletions affect viral pathogenicity, several observations indicate that the alterations have played an important role in the process leading to the tumor formation in these three animals and that they are likely to result from a selection for more-pathogenic viral structures than the original viral forms. (i) Although distinct, the alterations of the three provirus types followed a common pattern, i.e., small deletions in the same area in the enhancer. (ii) One of the tumors with altered proviral structures came from the only mouse that developed a lymphoma upon inoculation with the very weakly pathogenic virus variant carrying mutations in both AML1 site I and site II. (iii) The altered proviral structure of the ATC series was found to be integrated in the c-*myc* gene in a manner often found for MLVs activating this gene in thymic lymphomas. (iv) From the Southern blots, the tumors appeared to be clonal or oligoclonal with respect to viral integrations. (v) The altered structures acted as stronger enhancers than their parental structures when measured in transient-transcription assays in T-lymphoma cells. (vi) Mutations of the NF1 sites did not impair the pathogenicity of SL3-3.

The deletions did not seem to create new transcription factor binding sites. They therefore probably acted by removing binding sites for one or more negatively acting factors. Since the enhancer strength increased twofold when the NF1 sites were mutated, we believe that the main function of the deletions is to abolish the function of the NF1 site. This notion is corroborated by the finding that NF1-mutated SL3-3 does not show reduced pathogenicity, in contrast to what is known for Moloney MLV. This shows that the NF1 sites are not necessary for viral replication and tumorigenicity in mice but does not rule out a possible negative contribution from the NF1 sites during the tumorigenic process, since such an effect could well be masked by the potency of the wild-type virus. Also, even though the NF1 sites presumably reduce the replicative potential of SL3-3 in the target cells, the sites may play an important stimulatory role in cell types other than the T cells, perhaps a cell type that the virus acts in at an early stage of the pathogenic process (18, 67). Our finding that mutation of the NF1 sites severely reduces the expression of the reporter constructs in NIH 3T3 cells together with a previously noted reduction in other cell lines (45) show that the NF1 sites do have a positive-acting effect in some cell types.

Several factors with the potential to down-regulate transcription could be imagined to bind the NF1 site. One possibility is that a form of NF1 binds to the site. Although NF1 has generally been considered an ubiquitously expressed activating factor, a more-complex picture has recently begun to emerge. The four NF1 genes in mammals and chicken are all alternatively spliced in a manner conserved across species boundaries (28). All NF1 polypeptides contain a highly conserved N-terminal region containing the DNA-binding and dimerization domain. However, they differ in the C-terminal region containing the proline-rich activation domain (28, 56). Moreover, the NF1 polypeptides form heterodimers, are found in various amounts in various cell types, and have been reported to be implicated in specific gene regulation in several different types of tissues (3, 10, 20, 26, 38). A potential therefore exists for

subtle regulatory functions by the NF1 complex. Products of the two NF1 genes that as yet have been characterized to some extent, NF1-C and NF1-X, indeed show differential transactivating abilities (37, 56), and one NF1-X gene product has even been shown to down-regulate the enhancer of human papillomavirus type 16 (4). Thus, it is possible that a version of the NF1 complex with a down-regulatory effect is present in the T-lymphoid cells. The possible regulation of SL3-3 by NF1 could also be dependent upon the transformed state of the host cells and thus could change during the process of tumor development, since the level of NF1 expression has been reported to be regulated by several factors implicated in tumorigenesis such as transforming growth factor β (1), Ha-Ras (41), and c-Myc (70), the latter being a frequent insertional target of SL3-3 (23, 40, 63).

One previous example of a naturally occurring NF1 mutant has been reported regarding the feline leukemia virus which in many ways is comparable to SL3-3. The finding of this virus correlated with the finding of a reduced level of NF1 in the feline leukemia virus-induced tumor cells (54). If the level of NF1 is down-regulated in the target cells of SL3-3 in the process of tumor formation, a factor other than NF1 could be imagined to bind the NF1 site. One possible candidate for such a factor is histone H1. Histone H1 can act as a general repressor and has been reported to be implicated in transcriptional regulation by NF1 (16) and to be able to bind to the NF1 site in the SL3-3 enhancer (45).

The three tumors all contained viral structures with an additional 72-bp repeat element. Generation of additional repeat elements during the course of pathogenesis has been observed previously regarding SL3-3 (40) and other MLVs (7, 61). Proviruses with variations in the number of repeat elements are generally found in more than half of the examined murine tumors induced by SL3-3 or the closely related Akv MLV (17). These proviruses most often have gained or lost one repeat element or more rarely gained two. In light of this, it is not surprising that we find in the same tumor the same deletion in more than one U3 version differing by the number of repeats. The fact that we frequently find variations in the number of repeats but only rarely see the NF1 site deletions indicates that the two types of alterations do not occur with the same frequency. The deletions seem to represent rare events, which are presumably being given time to occur and be effective because of the prolonged latencies caused by the core mutations. The repeat variations on the other hand occur much more frequently and in all likelihood arose after the creation of the deletions in the cases discussed here. This is in agreement with the notion that the reverse transcriptase has an inherent propensity to skip or create small repeat structures (11), perhaps as a consequence of its ability to perform jumps—a necessary feature of the process of reverse transcription. Interestingly, the various repeat numbers also indicate that the viruses with the enhancer deletions were replicating in the mice, thus strengthening the argument for an active role in the disease induction played by the altered structures. In line with this reasoning, our transfection experiments show that the deletions contribute more to the increased enhancer effect than do the extra repeat elements.

Why is the NF1 site of Moloney MLV important for the pathogenicity of this virus when the corresponding NF1 site of SL3-3 does not contribute to the pathogenicity? The NF1 sites of the two viruses are identical except for a 1-bp difference. This difference may be of some importance, but SL3-3 provirus from tumors would then be expected to frequently adopt the Moloney MLV sequence, something which we have not seen to occur. Other factors interacting with the enhancer repeats could also play a role for the usage of the NF1 sites. Moloney MLV and SL3-3 are similar in the region known as the enhancer framework (19) encompassing the core, Ets, NF1, and the overlapping GR and ALF1 sites, but SL3-3 does not contain the LVc site which however is of only moderate importance for the pathogenicity of Moloney MLV (59). However, they differ in the remaining areas of the repeat region in which Moloney MLV contains a second NF1 site and the LVa binding site, instead of the AML1 core site II and Myb site (19, 58). Apart from the possibility that the various factors may interact differently with one another in the two enhancer regions, this also gives an overall impression of the SL3-3 enhancer as more specialized for T-lymphomagenic expression than the Moloney MLV enhancer. This again may point to possible differences in the mechanism of tumor induction between the two viruses. Perhaps Moloney MLV might be more dependent than SL3-3 upon replicating in cell types other than T lymphocytes, cell types where NF1 could be relatively more important for viral expression.

ACKNOWLEDGMENTS

We thank Annette Balle Sørensen for helpful discussions and technical advice. The technical assistance of L. Højgaard, A. Appold, A. Nickl, and E. Samson is gratefully acknowledged.

This project was supported by the Danish Cancer Society, the Karen Elise Jensen Foundation, the Danish Research Academy, the Danish Natural Sciences Research Council, The Danish Biotechnology Programme, the Leo Nielsen Foundation, European Commission contracts CT-950100 (Biotechnology) and CT-950675 (Biomed-2), the Swedish Cancer Society, the Swedish Natural Science Research Council, and the Cancer Research Foundation, Umeå, Sweden.

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